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13. ABSTRACT *(Maximum 200 words)*

Polyphenolic compounds in green tea have been implicated in the prevention of cancer, but their effects on cell signalling pathways are unknown. The current work was directed towards defining how such compounds inhibit cell proliferation and whether they inhibit oncogenic c-neu induced breast cancers in mice. Using the EGF dependent growth of MCF10A epithelial cells as a model, we found that the major polyphenol in green tea, EGCG, inhibited progression through the late G1 cell cycle restriction point, but did not affect early steps in receptor tyrosine kinase signalling. Rather, EGCG sustained the EGF-dependent expression of the $p21^{CIP1}$, inhibitor of cyclin-dependent kinases and impaired phosphorylation of the retinoblastoma protein, pRB. This finding defines a novel way in which chemopreventive agents such as EGCG may inhibit mitogenic signals. In vivo studies failed to detect an inhibitory effect of green tea or tea polyphenols on c-neu induced mammary tumorigenesis in mice, suggesting that c-neu signals in a way that resists the effects of such compounds.

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David Coleman 4/25/99

PI - Signature Date

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INTRODUCTION

Green tea polyphenols (GTPs) are potentially useful for delaying the onset of breast cancer, since they suppress tumorigenesis in rodents, they inhibit tumor cell proliferation *in vitro* and *in vivo*, and they are apparently safe natural products (1). However, since the mechanism through which GTPs suppress tumorigenesis is not known, it is unclear how to maximize their efficacy to prevent cancer or how to identify or develop compounds with improved efficacy. Accordingly, the current studies were undertaken to define the mechanism through which green tea polyphenols (GTPs) inhibit cell proliferation, as well as to evaluate their ability to suppress a genetically defined mammary tumor in mice.

Studies to determine how GTPs inhibit breast tumor cell proliferation initially evaluated the hypothesis that GTPs impair mitogenic signalling by receptor tyrosine kinases (RTKs) by preventing the accumulation of hydrogen peroxide (H_2O_2) and thus sustaining the activity of tyrosine phosphatases that otherwise diminish RTK phosphorylation and activity. This hypothesis was supported by evidence that GTPs reduce H_2O_2 levels *in vivo*, that H_2O_2 can mediate mitogenic signalling (2,3), that H_2O_2 promotes tyrosine phosphorylation of the EGF and insulin receptors (3-7), and that H_2O_2 can inhibit tyrosine phosphatases (8). Additional studies were to focus on the effects of GTPs on the proliferation and RTK phosphorylation of non-transformed MCF10A human mammary epithelial cells, as well as on MCF-10A derivatives expressing human erbB-2 (MCF10A/*erbB-2*) or a constitutively active rat c-*neu* oncogene (MCF10A/c-*neu*) (9,10).

As described in Results, we found that the major polyphenol in green tea, epigallocatechin gallate (EGCG), inhibited proliferation of MCF10A, MCF10A/*erbB-2*, and MCF10A/c-*neu* cells to similar extents. Moreover, EGCG had no effect on the transient tyrosine phosphorylation of erbB-2 in EGF-stimulated MCF10A or MCF10A/c-*neu* cells (see below). These findings argued against the hypothesis that GTPs impaired RTK tyrosine phosphorylation. Accordingly, we reappraised our strategy for defining GTP anti-mitogenic effects. Rather than focus on selected early signalling events such as H_2O_2 production and RTK phosphorylation, we defined the effects of EGCG on cell cycle progression as a whole, and showed that it blocked progression from the G1 to the S phase of the cell cycle. We then defined the time in G1 when MCF10A cells were most sensitive to EGCG, and determined that cells were most sensitive in mid G1 and lost sensitivity as they progressed through the late G1 restriction point. We then evaluated the effects of EGCG provided to mid G1 MCF10A cells on regulators of G1 cell cycle progression, and found that EGCG sustained the EGF-induced expression of the CDK inhibitor protein, $p21^{CIP1}$. As described in the Discussion, these studies define a novel regulatory mechanism for cell growth inhibition by GTPs and provide leads to define the precise molecular effects of EGCG leading to growth inhibition.

The *in vitro* studies were performed along with studies to evaluate the effects of GTPs on mammary tumorigenesis in MMTV/c-*neu* transgenic mice (11). These mice express constitutively active c-*neu* and develop mammary adenocarcinoma *in situ* in all transgene-expressing mammary tissue, with palpable tumors formed between 11 and 14 weeks in parous females. The rapid tumor development, nearly uniform timing of tumorigenesis, and genetic identity of the mice make them appropriate for studying the effects of GTPs on c-*neu*-induced oncogenesis. In the event that green tea or GTPs were to inhibit tumorigenesis, it was intended to determine whether such agents affected RTK phosphorylation and cell proliferation or survival. Whereas pilot studies indicated that tumor burden was diminished in MMTV/c-*neu* mice by orally administered green tea, neither green tea, decaffeinated green tea, nor GTPs inhibited tumorigenesis in subsequent analyses. Possible explanations for this are presented in the Discussion.

BODY

EXPERIMENTAL METHODS

Cell Culture and Treatments. MCF10A cells were maintained as described previously (12) in DMEM:F12 (1:1) and 5% horse serum supplemented with 4 mM L-glutamine, 100 ug/ml penicillin/streptomycin, 20 mM HEPES, 10 ug/ml insulin, 0.5 ug/ml hydrocortisone, 100 ng/ml cholera toxin, and 20 ng/ml EGF. For all experiments, cells were seeded into a serum free media described previously (13) that was supplemented with 2% horse serum, referred to as 'plating media'. Serum free media consisted of the DMEM:F12 base described above, supplemented with 1 mg/ml BSA, 5 ug/ml transferrin, 20 ng/ml sodium selenite, 1 ug/ml hydrocortisone, and 1 mM ethanolamine. Cells were cultured in plating media for two days before each experiment. Experiments were performed in serum free media to which was added either nothing or the appropriate concentration of EGF and/or EGCG. For experiments measuring S phase entry, 30 uM 5-bromo-2-deoxyuridine (BrdU), 30 uM 2-deoxycytidine, and 10 uM 5-fluorodeoxyuridine were added to the media.

Cell Proliferation Assay. Cells were seeded into 6-well plates and cultured for 2 days in plating media. Media was then replaced with serum free media containing 5 ng/ml EGF without or with EGCG to a final concentration of 25 uM, 50 uM, or 100 uM. Cells were cultured for three additional days, and then triplicate wells were counted with a hemocytometer using trypan blue to exclude dead cells.

Analysis of S-phase Entry. Detection of BrdU and propidium iodide was by flow cytometry using standard protocols provided by Becton-Dickinson (San Jose, CA). Anti-BrdU and fluorescein conjugated secondary antibodies were from Becton-Dickinson. Briefly, BrdU labelled cells were harvested and fixed in ice cold 70% ethanol for 30 min. The remaining incubations were all performed at room temperature. DNA was denatured by treatment with 2N HCl containing 0.5% Triton™ X-100 followed by neutralization with 0.1 M sodium tetraborate. BrdU was detected by indirect immunofluorescence with a mouse monoclonal antibody against BrdU (20 ul/ml) followed by a fluorescein conjugated goat anti-mouse secondary antibody (80 ul/ml). After each antibody incubation, cells were washed in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.5% Tween 20. Total DNA was stained with propidium idodide (10 ug/ml). Nuclei were analyzed by two-color flow cytometry on a Becton-Dickinson FacsCalibur using 10^4 singlet gated events per sample. Singlet gating was to exclude aggregates from analysis. Quantitation was done using CellQuest™ software (v. 3.1f).

Sensitivity to EGCG and EGF During G1: Cells were seeded into 10 cm tissue culture dishes (Corning, Inc.) and cultured for 2 days in plating media. Experiments were performed in serum free media. For experiments requiring EGCG exposure during a specified interval of time, cells were stimulated with EGF and EGCG was added directly to individual plates at the appropriate time. Two hr after the addition of EGCG, the media containing EGCG was replaced with media containing EGF alone. For each interval, the media was also changed in an untreated (EGF only) plate as a control for possible effects of replacing the media during G1 phase. S phase entry for each interval was normalized to that of the EGF control whose media had been replaced at the same time. For experiments not requiring a defined interval of treatment, EGCG was added directly to the appropriate plates and the media was not changed during the experiment. One EGF control was used to normalize in these experiments. For the EGF withdrawal experiment, the media was replaced with serum free media lacking EGF at the appropriate times. Data was normalized to one control in which EGF was present continuously and the media was never changed. All experiments were performed in duplicate for each time point or treatment.

Western Blotting. Cells were lysed in 150 mM NaCl, 50 mM Tris, 1 mM EDTA, 5% sodium deoxycholate, and 10% Nonidet P-40 supplemented with pepstatin (10 ug/ml), sodium vanadate (1 mM), leupeptin (50 ug/ml), aprotinin (20 ug/ml), phenylmethylsulfonate (100 ug/ml) and nitrophenyl phosphate (15 mg/ml). Total protein was quantitated using a BioRad DC assay kit and equal amounts of protein were loaded onto a denaturing polyacrylamide gel (37:1). Indirect immunoblotting was performed using the following primary antibodies according to manufacturer's instructions: cyclin D1 (ms-210) and $p21^{CIP1}$ (ms-230) from LabVision Corp. (Fremont, CA); cyclin E (sc-247), $p27^{KIP1}$(sc-528), and p53 (sc-126) from Santa Cruz Biotechnology (Santa Cruz, CA); pRB (14001A)from Pharmingen (San Diego, CA); ERK1/2 (9102) and phospho specific ERK1/2 (9101-S) from New England Biolabs (Beverly, MA). The appropriate horse radish peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Detection was with an enhanced chemiluminescence kit from Kirkegaard&Perry (Gaithersburg, MD).

Effect of green tea, decaffeinated green tea, and green tea polyphenols (GTPs) on mammary tumorigenesis. 4-week-old female MMTV/c-*neu* transgenic mice were obtained from Charles River Laboratories and were provided with increasing concentrations of a standard green tea, decaffeinated green tea, and GTPs (Lipton Co.) in their drinking water until reaching 1% in one week. At 25 weeks of age, mice were sacrificed, mammary tissue was isolated, and either fixed and sectioned histologically or prepared for whole mount staining.

RESULTS

Effect of EGCG on MCF10A cell proliferation and S phase entry. To identify events that were sensitive to EGCG, we first determined the range of concentration that gave a dose-dependent inhibition of proliferation. MCF10A cells were growth arrested by plating in low serum and culturing for two days. This was sufficient for at least 90% of cells to accumulate in the G0/G1 phase of the cell cycle as measured by flow cytometry (data not shown). The plating media was then replaced with serum free media containing EGF without or with EGCG, and the cells were allowed to grow for three additional days. As shown in Fig. 1, EGCG inhibited MCF10A cell proliferation in a dose-dependent manner between 25 and 100 uM. Dead cells accounted for less than 5% of the total in any of the treatment groups and there was no increase in dead cells with increasing concentrations of EGCG, indicating that the lower cell number in EGCG treated cultures was not due to cytotoxicity.

The mitogenic signal initiated by EGF culminates with passage through the late G1 restriction point and the commitment of cells to enter S phase (14). Accordingly, if EGCG was interfering with EGF signaling, then it would also be expected to inhibit S phase entry. To examine this, cells synchronized in G0/G1 were stimulated with EGF in serum free medium containing the nucleotide analogue, 5-bromo-2-deoxyuridine (BrdU), either in the presence or absence of 40 uM EGCG. The cells were analyzed approximately 20 hours later when 25-30% of cells had entered S phase but none had yet completed mitosis. The population of cells which had entered S phase was identified by flow cytometry as shown in Fig 2A, boxed regions. EGCG inhibited the S phase entry of EGF stimulated MCF10A cells by approximately 50% (Fig 2A). Increasing concentrations of EGCG up to 150 uM progressively inhibited S-phase entry (Fig 2B) without significant cytotoxicity except at the highest dose.

Effect of c-*neu* oncogene expression on MCF10A sensitivity to EGCG. We then determined whether expression of the c-*neu* oncogene altered the sensitivity of MCF10A cells to EGCG. Growth arrested MCF10A or MCF10A/c-*neu* cells were stimulated with EGF in the presence or absence of 40 uM EGCG. Whereas a greater proportion of MCF10A/c-*neu* cells entered S phase, they were equally susceptible to EGCG, displaying about 50% of S phase entry as compared to cells treated with EGF alone. The effect was dose dependent in the range of 30 -

90 uM (data not shown). Thus, EGCG impaired S phase entry in normal and in oncogene-expressing mammary tumor cells to similar extents.

Effect of EGCG on erbB-2 tyrosine phosphorylation. In order to address whether the anti-proliferative effect of EGCG was mediated through decreased RTK phosphorylation, we evaluated the effect of EGCG on erbB-2 tyrosine phosphorylation. Growth arrested MCF10A or MCF10A/c-*neu* cells were stimulated with EGF in the presence or absence of 40 uM EGCG, and cell lysates were prepared at 15, 30, 60, and 120 minutes. Lysates were then subjected to immunoprecipitation with anti-erbB-2 antibody, and the immunoprecipitates were subjected to Western blotting with either anti-phosphotyrosine or anti-erbB-2 antibody. The results showed a transient increase in phosphotyrosine associated with immunoprecipitated erbB-2, and a slight decline in overall levels of erbB-2 over the two hours of the experiment. However, there was no difference in phosphotyrosine associated or total erbB-2 over this time in EGCG treated versus untreated samples in either MCF10A or MCF10A/c-*neu* cells (data not shown). These findings indicated that EGCG did not alter the early EGF-depedent activation of the erbB-2 RTK. Further experiments suggested that EGCG also did not affect signalling events that are known to occur subsequent to EGFR or erbB-2 phosphorylation, as EGCG had no effect on the EGF-dependent activation of the Erk1/2 MAP kinases or upon the EGF-dependent decrease in $p27^{KIP1}$ (see below). Because of theis, we decided to focus on the cell cycle regulatory events that were being affected by EGCG, with the idea that identification of such events would allow us to work backwards from the affected cell cycle signalling events to the precise molecular effects of EGCG.

Effect of EGCG on Progression through the late G1 restriction point. To determine the time in G1 when MCF10A cells were most sensitive to EGCG, we added EGCG at various times after stimulation with EGF, either for 2 hr intervals (Fig. 3A) or for the duration of the experiment (Fig. 3C), as described in Materials and Methods. In both cases, EGCG maximally inhibited S phase entry when it was administered during mid G1, at least 4 hrs after EGF. The effect was dose-dependent in the range of 10-100 uM EGCG (Fig. 3B). The effect of EGCG provided during the intervals 6-8 or 8-10 hrs after EGF was somewhat greater than with continuous treatment begun at 6 or 8 hrs, presumably as a consequence of changing the media after each interval. Notably, the effect of EGCG added at 10 hrs after EGF was significantly diminished from that of earlier times (Fig. 3C). This decreased sensitivity to EGCG occurred in late G1, since the first cells entered S between 12-13 hrs after EGF stimulation (data not shown).

The increased sensitivity to EGCG during mid G1 as well as the decreased sensitivity in late G1 suggested that EGCG was interfering with an event that was important for the late G1 restriction point transition. To evaluate the timing of the transition from mitogen dependence to mitogen independence that characterizes the restriction point, EGF was removed from the media at times during G1 progression. As shown in Fig. 3D, the proportion of MCF10A cells entering S phase increased progressively as EGF was allowed to remain in the media for longer times, with between 40-60% of the normal S phase entry occurring when EGF was withdrawn after 10 hrs. Thus, the majority of MCF10A cells lost their requirement for EGF near this 10 hr time point, about the same time as they lost sensitivity to EGCG, suggesting that the cells became insensitive to EGCG as they approached or passed through the restriction point.

Effect of EGCG on phosphorylation of the retinoblastoma protein. The progressive phosphorylation of the retinoblastoma protein (pRB) during G1 is a growth factor dependent process whose disruption would be expected to inhibit passage through the restriction point (15). To determine whether EGCG treatment attenuated pRB phosphorylation, growth arrested cells were restimulated with EGF and EGCG was added during mid G1, when it exerted maximal inhibition of S phase entry. The electrophoretic mobility of pRB, which is an indication of its phosphorylation state, was analyzed by western blotting (Fig. 4). In cells treated with EGF alone, phosphorylated forms of pRB (ppRB) were evident at 5 hours and continued to accumulate at later

times (Fig. 4, lanes 2, 3, 5). In contrast, providing EGCG at 5 hours reduced the level of ppRB that was present at 8 and 12 hours to below that which was present at the 5 hour time point (compare lanes 3 and 5 with lanes 4 and 6). This effect was transient, since by 24 hours the amount of ppRB in treated cells approximated that of untreated cells (compare lanes 7 and 8). Thus, addition of EGCG during mid G1 inhibited the accumulation of ppRB, an effect that would be expected to delay passage through the restriction point as well as entry into S.

Effect of EGCG on the expression of cell cycle regulatory proteins. The phosphorylation of pRB requires the activity of the G1 cyclin dependent kinases (CDKs), which in turn are regulated by positive regulatory subunits termed cyclins and by inhibitory proteins or CDKIs. Thus, to understand the basis for the inhibition of pRB phosphorylation, we examined the effect of EGCG on these regulators of G1 CDK activity.

Effect of EGCG on the accumulation of G1 cyclins: The mitogen dependent accumulation of D-type cyclins during early and mid G1 activates CDK4 and CDK6 and is required to initiate pRB phosphorylation (32). Since EGCG reduced the amount of ppRB in mid G1, we investigated whether it also inhibited the accumulation of D-type cyclins. In preliminary experiments, MCF10A cells expressed cyclin D1 but not detectable levels of cyclins D2 or D3 (not shown). In response to EGF alone, cyclin D1 expression increased substantially within 4 hours (Fig 5A). Addition of EGCG concurrently with EGF reduced the accumulation of cyclin D1 during this time. This effect was dose-dependent in the range of 25-100 uM EGCG (Fig. 5B) and was transient, since at later times (10 h) cyclin D1 expression in EGCG treated cells approximated that of untreated cells (data not shown).

We next examined the expression of cyclin D1 under conditions where EGCG had maximally inhibited S phase entry. EGCG was administered during mid G1, beginning 5 hours after EGF addition. Examination of protein lysates taken at subsequent times showed little change in cyclin D1 expression with EGCG treatment (Fig 6A). Thus, while there was a decrease in cyclin D1 expression when EGCG was administered along with EGF, there was not a substantial effect when it was administered during mid-G1, a time of maximal sensitivity to EGCG. This result suggested that although EGCG could inhibit the initial accumulation of cyclin D1, this effect alone was not sufficient to explain its inhibition of S phase entry.

The activity of CDK2-cyclin E during mid/late G1 is required for the complete phosphorylation of pRB and S phase entry (16). In many cell types, the expression of cyclin E increases in late G1 coinciding with activation of CDK2. Accordingly, we examined the expression of cyclin E to determine whether it was altered by the presence of EGCG. As shown in Fig. 6B, cyclin E expression increased during the first 5 hours after EGF stimulation (compare lanes 1 and 2) and then remained relatively constant through the remainder of G1 (5-12 h). This expression of cyclin E was not affected by EGCG administered during mid G1 (5 h). These results indicated that the inhibition of pRB phosphorylation by EGCG was not mediated through a decrease in the amount of either cyclin D1 or cyclin E proteins.

Effect of EGCG on $p21^{CIP1}$ and $p27^{KIP1}$ expression: In order for catalytically active CDK-cyclin complexes to form during G1, an inhibitory threshold set by the levels of $p21^{CIP1}$ and $p27^{KIP1}$ must be overcome (17). This is accomplished through a combination of increased cyclin expression and a decline in the expression of $p21^{CIP1}$ and $p27^{KIP1}$. The failure to repress the expression of $p21^{CIP1}$ and/or $p27^{KIP1}$ would be expected to prevent the timely phosphorylation of pRB. Accordingly, we examined whether EGCG affected the expression of $p21^{CIP1}$ and $p27^{KIP1}$ during G1 progression.

As shown in Fig. 7A, the expression of $p21^{CIP1}$ was rapidly increased after EGF stimulation. In the absence of EGCG, protein levels peaked in about 2 hours, then decreased to basal amounts by 4 hours (Fig. 7A, lanes 7 and 8). When EGCG was administered concurrently

with EGF, the initial $p21^{CIP1}$ expression was slightly higher than that observed with EGF alone (compare lanes 3, 5, and 7 with lanes 2, 4, and 6). In addition, at 4 hours, $p21^{CIP1}$ expression was maintained in EGCG treated cells while in untreated cells the expression of $p21^{CIP1}$ had returned to low levels (Fig. 7A, compare lanes 8 and 9). The increased expression of $p21^{CIP1}$ with EGCG treatment was dose-dependent in a range of 25 to 100 uM EGCG (Fig. 7B).

Since EGCG treatment both increased the early expression of $p21^{CIP1}$ and sustained its expression at times when it would otherwise be low, we examined the possibility that EGCG was able to induce $p21^{CIP1}$ expression in the absence of EGF. As shown in Fig. 7C, the low basal expression of $p21^{CIP1}$ in growth arrested MCF10A cells was not increased 2, 4, 6, or 8 hrs after the addition of 50 uM EGCG. As a control, EGF treated cells showed high $p21^{CIP1}$ expression at 2 h (Fig. 7C lane 3).

We next examined the expression of $p21^{CIP1}$ when EGCG was administered at 5 hours after stimulation with EGF, during the time of maximal sensitivity to EGCG. Strikingly, 3 hours after addition of EGCG, $p21^{CIP1}$ expression had increased, and it remained higher through the remainder of G1 (Fig. 6C, compare lanes 3 and 5 with lanes 4 and 6). In addition, a slower migrating $p21^{CIP1}$ species formed in EGF stimulated cells at 24 hrs but was not present in EGCG treated cells (compare lanes 7 and 8). Since this species likely represents a phosphorylated $p21^{CIP1}$ isoform that associates with CDK-cyclin A during G2/M (18,19), its absence in EGCG treated cells suggests that cell cycle progression was delayed.

In contrast to the effect seen with $p21^{CIP1}$, EGCG treatment during mid G1 did not affect the expression of $p27^{KIP1}$ (Fig 6D). The expression of this CDK inhibitor was high in early G1 and decreased substantially between 5 and 12 hours, regardless of whether cells were treated with EGCG. The expression of $p27^{KIP1}$ at 24 hours was somewhat higher in EGCG treated cells, most likely as a consequence of delayed G1 progression (Fig 6D, compare lanes 7 and 8). Thus, EGCG treatment of G1 MCF10A cells disrupted the down-regulation of $p21^{CIP1}$ while having no immediate effect on the repression of $p27^{KIP1}$.

Effect of EGCG on p53 expression and ERK1/2 activation during G1. An increase in $p21^{CIP1}$ could play a significant role in the inhibition of S-phase entry by EGCG by impairing pRB phosphorylation and passage through the restriction point. Therefore, we examined several possible mechanisms through which EGCG treatment might lead to increased $p21^{CIP1}$.

The p53 protein accumulates and induces $p21^{CIP1}$ expression in response to DNA damage and oxidative stress (20,21). Since many flavonoids, including EGCG, can behave as oxidants (22,23), and since MCF10A are wild type for p53 (24), the accumulation of $p21^{CIP1}$ in response to EGCG could be mediated through a p53 dependent oxidative stress response. To examine this, we determined whether p53 protein levels were altered by EGCG treatment during G1 progression. As shown in Fig. 6E, the expression of p53 remained relatively constant during G1 and was not changed by a mid G1 addition of EGCG. However, addition of EGCG at this time was followed by increased $p21^{CIP1}$ expression (Fig. 6C), arguing against a role for p53 in the EGCG induced accumulation of $p21^{CIP1}$.

$p21^{CIP1}$ is also induced by a p53 independent mechanism in response to serum and activated Ras or Raf. The induction of p21 by high level Ras or Raf signaling and possibly by serum growth factors, depends upon activation of the extracellular regulated kinases 1 and 2 (ERK1/2), which are the terminal kinases in the growth factor initiated MAP kinase pathway (25,26).

Accordingly, we investigated whether the increased expression of $p21^{CIP1}$ in response to EGCG coincided with ERK1/2 activation in MCF10A cells. To do so, we identified the active, phosphorylated forms of ERK1/2 by western blotting with antibodies specific to these species. As shown in Fig. 8A, ERK1/2 was rapidly phosphorylated after EGF addition. However, the EGF induced activation of ERK1/2 was transient, having peaked and then subsided to basal levels between 1 and 3 hours, as expected for an ERK1/2 mitogenic signal (27). EGCG (25 or 100 uM) added either concurrently with EGF or during mid G1 had no significant effect on ERK1/2 phosphorylation (Fig. 8A, B). Thus, EGCG treatment did not affect the initial activation of ERK1/2 or the activity of the kinases later in G1, suggesting that neither the EGCG mediated decrease in cyclin D1 expression nor the increase in $p21^{CIP1}$ was effected through changes in ERK1/2 activity.

Effect of green tea, decaffeinated green tea, and green tea polyphenols (GTPs) on mammary tumorigenesis in MMTV/c-*neu* transgenic mice

A pilot study described in the Preliminary Results of the original proposal showed that of three control (water drinking) mice, one had developed palpable masses at 20 weeks and two lacked overt abnormalities at 25 weeks. In contrast, none of the five mice drinking 1% lyophilized green tea (LGT) had formed palpable masses by 25 weeks, but these also exhibited substantial weight loss. Subsequent histologic analysis of the mice showed that the tea drinking mice developed fewer tumors than the controls (Table 1a). However, the necropsy also revealed evidence of gastrointestinal dysfunction in the tea group, with substantial amounts of undigested foods in the upper GI tract, which seems likely to have contributed to weight loss. This effect of 1% tea has been observed previously in some but not all strains of mice and may relate to the age and rate at which tea drinking is initiated (personal communication), and seems likely to have contributed to the weight loss associated with tea drinking. The GI toxicity and weight loss raised the possibility that the antitumor effect of tea might not have been a direct consequence of polyphenols, but could have resulted from changes in overall caloric balance.

To address these concerns, a second study was performed in which tumor formation in MMTV/c-*neu* mice was quantitated after drinking water (10 mice), 0.8% LGT (6 mice), decaffeinated lyophilized green tea (dLGT) (6 mice), or GTPs (5 mice). In this study, there was not substantial weight loss among the LGT population, presumably due to the decreased tea concentration and/or more progressive weaning onto tea. The results showed no significant anti-tumor effect of any of the tea preparations (Table 1B). Finally, two additional cohorts of mice which were progressively weaned onto 1% LGT, starting at 5 weeks of age, or controls provided with water were analyzed for the presence of hyperplastic nodules in mammary tissue whole mounts. In these mice, there was no significant weight difference between the two populations and there was a trend towards an increased occurrence of such nodules in 1% LGT drinking mice (Table 1C). However, this trend appeared not to be statistically significant, due to the great variability in tumor nodules per mouse in each group and the low number of mice used. In summary, these results indicate that tea is not an effective chemopreventive agent in MMTV/c-*neu* mice.

Table 1. Effect of Orally Administered Tea Preparations on Tumorigenesis in MMTV/c-*neu* Transgenic Mice

A.

# of Tumors	
Water	1% LGT
5	18
3	16
2	6
0	
Average per mouse	
2.5	10

B.

# of Tumors			
Water	.8% LGT	dLGT	GTP
11	10	20	17
9	9	7	17
9	6	6	10
8	6	3	6
8	6	3	3
6	3		
5			
3			
3			
0			
6.2	6.7	7.8	10.6

C.

Cohort 1 # Hyperplastic Nodules		Cohort 2 # Hyperplastic Nodules	
Water	1% LGT	Water	1% LGT
24	28	10	35
20	25	10	25
7	24	9	10
5	6	6	9
4	4	5	9
		4	3
		4	2
Average per mouse			
12.0	17.4	6.9	13.3

DISCUSSION

The studies described in this report have addressed the central goals of the original proposal: a) to decipher the mechanism through which the green tea polyphenol, EGCG, inhibits cell proliferation, and b) to determine whether tea polyphenols inhibit tumorigenesis in MMTV/c-*neu* mice.

A. Effects of EGCG on cell proliferation

The experiments that were directed to determine how GTPs inhibits cell proliferation focused on the hypothesis that such agents may inhibit RTK phosphorylation and signalling by diminishing cellular H_2O_2 levels. These studies were to involve:

Task 1) Determining the GTP concentrations needed to inhibit proliferation of MCF-10A and MCF-10A-derived cells treated with EGF, β-NDF, or IGF-I;

Task 2) Determining whether growth-inhibiting concentrations of GTPs also diminish tyrosine phosphorylation associated with the EGFR, erbB-2, or IGF1R; and

Task 3) Establishing the effect of growth inhibitory doses of GTPs upon H_2O_2 accumulation.

As described in Results, we have in the past year focused on the effects of the green tea polyphenol EGCG upon EGF dependent cell proliferation, but have not addressed its effects upon β-NDF or IGF-I dependent cell growth. This decision was based on the ability of MCF10A cells to grow with EGF as the only growth factor and on our finding that EGCG inhibited such proliferation. We reasoned that it would be appropriate, at least initially, to focus on the effects of a single GTP (in this case EGCG) upon a single growth factor induced mitogenic signalling pathway, and then to determine the effects of EGCG on other proliferative signalling pathways after one mechanism of EGCG-inhibition of proliferative was established.

To this end, the present studies have defined the EGCG concentrations needed to inhibit EGF induced proliferation of MCF10A and MCF10A/c-*neu* cells, and have demonstrated that the extent of growth inhibition by EGCG was similar for both cell types. In addition, we found that EGF-induced erbB-2 tyrosine phosphorylation and Erk1/2 MAP kinase activation were not affected by EGCG treatment. These findings argued against our initial hypothesis that tea polyphenols inhibit RTK phosphorylation and signalling by diminishing cellular H_2O_2 levels, and suggested that the mechanism of growth inhibition was common to the two cell types. Accordingly, we set out to better understand this mechanism, using the MCF10A cells as a model. Studies to evaluate the effect of EGCG on H_2O_2 accumulation have not been pursued, as there is not compelling evidence to link such effects to a potential H_2O_2-sensitive signalling event. However, the effect of EGCG on H_2O_2 accumulation will be evaluated should potential H_2O_2-sensitive signalling events become evident in the next year.

Using the EGF dependent growth of MCF10A cells as a model, we found that EGCG maximally inhibited S phase entry when it was administered during mid G1. Sensitivity to EGCG was lost later in G1, corresponding to the transition from EGF dependence to EGF independence that defines restriction point passage.

The requirement for growth factors such as EGF during most of G1 may be explained by the need to maintain pRB in a partially phosphorylated state (16). The complete, or hyperphosphorylation of pRB in late G1 is dependent on this prior phosphorylation, and is tightly linked to passage through the restriction point. Significantly, EGCG prevented the partially phosphorylated form of pRB from being maintained during G1 progression. This strongly argues that EGCG inhibited S phase entry by inhibiting pRB phosphorylation and delaying restriction point passage.

The phosphorylation and inactivation of pRB depends upon the activity of CDK4/6-cyclin D in mid G1 followed by CDK2-cyclin E in late G1 (16). Thus, to inhibit the mid G1 phosphorylation of pRB, EGCG most likely impaired the activity of these kinases. This is supported by our results showing that EGCG both decreased the expression of cyclin D1 and increased the expression of the CDK inhibitor $p21^{CIP1}$. Of these effects, the increased expression of $p21^{CIP1}$ was probably more relevant to inhibition of S phase entry, because it was induced by a mid G1 addition of EGCG. Although addition of EGCG at this time gave the maximal inhibition of S phase entry, it had no effect on the expression of cyclin D1.

In addition to the effects of EGCG on MCF10A cells shown here, the proliferation or survival of other cell lines has also been shown to be inhibited by EGCG as well as by other flavonoids (23,28-34). Two of these, genestein and silymarin, also induced a cell cycle arrest that was accompanied by a p53-independent increase in $p21^{CIP1}$ (31,32). In addition, genestein increased the expression of $p21^{CIP1}$ in tumor cell xenografts, coinciding with a decrease in tumor size (32,35). These findings suggest that the induction of $p21^{CIP1}$ by some flavonoids *in vitro* may be relevant to their chemopreventive activity.

The mechanism through which EGCG increased the expression of $p21^{CIP1}$ is not known. Our results do not support a role for either p53 or ERK1/2 in this process since EGCG did not affect p53 expression or ERK1/2 activation during G1 progression. It was notable that in response to EGF, the expression of $p21^{CIP1}$ was transient, having been rapidly induced and then diminished. This suggests another explanation for the increase in $p21^{CIP1}$ observed with EGCG, namely that EGCG inhibited the decrease in $p21^{CIP1}$ that would normally have occurred. This possibility is supported by our results showing that EGCG by itself did not induce $p21^{CIP1}$ expression, but only sustained higher levels of $p21^{CIP1}$ after its initial induction by EGF.

$p21^{CIP1}$ has been shown to be induced by serum growth factors and by ectopically expressed Ras or Raf (21,26,36,37), yet the mechanisms regulating its repression during G1 in response to mitogenic stimuli are not well understood. Recent studies support a role for Rho proteins in the active repression of $p21^{CIP1}$ induced by Ras (38). Notably, activation of Rho is required along with Ras for G1 progression in response to serum and growth factors (39,40). In addition, Rho is required for transformation by oncogenic Ras and appears to be needed solely to counteract the Ras dependent induction of $p21^{CIP1}$ (38,41). Thus, Rho is an important regulator of mitogenic signaling by Ras and a plausible target for chemopreventive agents such as EGCG, which may sustain $p21^{CIP1}$ by inhibiting Rho. Of interest, EGCG inhibited the transformation of JB6 mouse epidermal cells (42) by inhibiting a pathway tightly linked to Rho signaling, the stress activated protein kinase (SAPK) pathway (43,44). While SAPK activation was not detected in response to EGF stimulation of MCF10A cells (unpublished observations), the ability of EGCG to inhibit transformation in JB6 cells and its ability to inhibit G1 progression in MCF10A cells may be mediated through a common target among the Rho family proteins.

Regardless of the precise mechanism through which EGCG sustains $p21^{CIP1}$ expression, its ability to do so enables it to affect a key mediator of the cell's response to Ras signaling. Activation of ERK can induce or inhibit proliferation in the same cells depending on the level of signaling (26,27). The sustained, high level activation of this pathway has been shown to induce a p53 independent expression of $p21^{CIP1}$ and to cause G1 arrest (26,45). However, in cases where high level Ras/ERK signaling results in proliferation and transformation, the repression of $p21^{CIP1}$ is also necessary (38,41). The results described here suggest that chemopreventive agents such as EGCG have the ability to interfere with this regulatory mechanism by sustaining the mitogen-induced expression of $p21^{CIP1}$.

B. Effects of green tea and GTPs on tumorigenesis in MMTV/c-*neu* mice

The second major goal of this proposal was to determine whether green tea or GTPs were able to inhibit tumorigenesis, cell proliferation or survival, and RTK phosphorylation in MMTV/c-*neu* mice. As shown in Results, we did not obtain evidence that green teas or GTPs were effective against the development of this tumor. However, preliminary studies with a small cohort of mice showed that under administration regimes associated with impaired GI function and substantial (~15%) weight loss, green tea impaired tumorigenesis. However, when green tea was provided at 0.8% or at 1.0% but with a slightly different administration routine, no tumor inhibitory effect was evident. These findings suggest that the initially observed effect of green tea (Table 1A), was likely to have been an indirect effect of GI toxicity and/or the associated caloric restriction, as opposed to a direct effect of GTPs. However, this possibility is highly tentative, since the effects have not as yet been replicated.

Due to the lack of effect of green tea or GTPs on MMTV/c-*neu* tumorigenesis in the studies conducted so far, it was determined to be appropriate not to further pursue the effects of EGCG on this tumor, such as by analysis of cell proliferation or survival, and RTK phosphorylation, as had initially been proposed.

CONCLUSION

Tea polyphenols inhibit tumorigenesis in numerous rodent models and have demonstrated *in vitro* effects on cell proliferation, apoptosis, and transformation. While the antioxidant activity of these compounds has been well established, the effects of tea polyphenols on cellular signaling pathways are not well understood. Here, we show that EGCG, the major polyphenol in green tea, sustained the expression of the cyclin dependent kinase inhibitor $p21^{CIP1}$ and impaired the G1 to S phase cell cycle transition in EGF stimulated MCF10A breast epithelial cells. EGCG showed a dose-dependent inhibition of S-phase entry between 10-100 uM with a maximal inhibition of 70% occurring when EGCG was added during mid G1. MCF10A cells lost sensitivity to EGCG in late G1 as they passed through the restriction point and acquired the ability to enter S phase without further growth factor stimulation. The inhibition of S phase entry by EGCG was accompanied by reduced phosphorylation of pRB and by a sustained increase in the expression of $p21^{CIP1}$. The ability of EGCG to increase $p21^{CIP1}$ levels depended upon prior induction of $p21^{CIP1}$ by EGF but did not require either the activation of ERK1/2 or increased expression of p53. These data suggest that EGCG interferes with the mid G1 decrease in $p21^{CIP1}$ that normally occurs in EGF stimulated MCF10A cells and thereby impairs pRB phosphorylation, progression through the restriction point, and S phase entry.

In additional studies, green tea or GTPs did not inhibit tumorigenesis in MMTV/c-*neu* mice. This lack of effect contrasts with the efficacy of such agents in numerous other mouse tumor model systems. Thus, it seems plausible that c-*neu* induced proliferative changes do not heighten a cell's sensitivity to EGCG or other polyphenols, as observed for other tumors. This possibility is supported by our *in vitro* finding that ectopic expression of c-*neu* did not increase sensitivity to EGCG. However, the expression of other oncogenes has been found by other to enhance sensitivity. By defining the mechanism of EGCG action *in vitro*, it may become feasible to determine how the expression some oncogenes increases sensitivity to EGCG, and thus to use green tea polyphenols and similarly acting agents in a rational way to prevent and possibly treat mammary and other cancers. For this reason, our ongoing studies will focus on the mechanism by which EGCG sustains the expression of $p21^{CIP1}$ in mitogen-stimulated cells.

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APPENDIX

FIGURE LEGENDS

Fig. 1. Effect of EGCG on MCF10A cell proliferation. Cells were growth arrested by culturing in low serum for two days and were then stimulated with EGF in serum free media containing either 0, 25, 50, or 100 uM EGCG. The number of viable cells was determined in triplicate on day three using a hemocytometer and trypan blue exclusion. Shown are average values with minimum and maximum counts indicated by error bars.

Fig. 2. Effect of EGCG on S phase entry of MCF10A cells. Cells arrested in G0/G1 were stimulated with EGF and S phase entry was quantitated at 18 h using two color flow cytometry. A, cells were treated with EGF (left) or EGF plus 40 uM EGCG (right). The x-axis represents propidium iodide staining, or DNA content, and the y-axis represents BrdU fluorescence intensity. B, cells were treated as in A except that the indicated concentration of EGCG was added along with EGF. Below is a graphical representation of the data.

Fig. 3. Sensitivity to EGCG and requirement for EGF during G1 progression of MCF10A cells. Growth arrested cells were stimulated with EGF and the average S phase entry at 22 hrs was determined for duplicate plates. Minimum and maximum values are represented by error bars. A, cells were exposed to EGCG for a 2 hr interval during G1, as indicated. B, the indicated concentration of EGCG was administered during an interval that was 4-6 hrs after stimulation with EGF. C, EGCG was administered at times after EGF as indicated. D, EGF was removed at the indicated times. Values were normalized to EGF without EGCG (A, B, and C), or to EGF present continuously in the media (D).

Fig. 4. Effect of EGCG on the phosphorylation of the retinoblastoma protein during G1. Growth arrested cells were stimulated with EGF without (-) or with (+) 50 uM EGCG added at 5 hrs. Total protein was collected at the indicated times and analyzed by western blotting with a pRB specific monoclonal antibody.

Fig. 5. Effect of EGCG on cyclin D1 expression during G1. A, cells were synchronized in low serum and stimulated with EGF in the presence (+) or absence (-) of 50 uM EGCG, or B, with the indicated concentration of EGCG. Total protein was collected at the indicated times and analyzed by western blotting with a cyclin D1 monoclonal antibody.

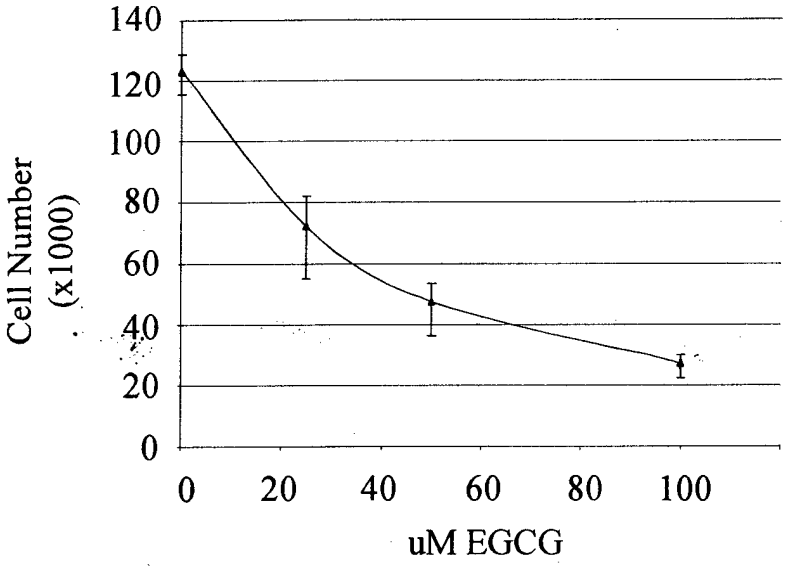
Fig. 6. Effect of EGCG addition during mid G1 on the expression of cell cycle regulatory proteins. Cells were synchronized in low serum and stimulated with EGF in the presence (+) or absence (-) of 50 uM EGCG added 5 hrs after EGF. Total protein was collected at the indicated times and analyzed by western blotting with antibodies to A, cyclin D1; B, cyclin E; C, $p21^{CIP1}$; D, $p27^{KIP1}$; and E, p53. The same protein lysates were used for all analyses.

Fig. 7. Effect of EGCG on $p21^{CIP1}$ during G1. A, cells were synchronized in low serum and stimulated with EGF in the presence (+) or absence (-) of 50 uM EGCG, or B, with the indicated concentration of EGCG. C, Cells which had been cultured in low serum for 2 days were incubated with (+) or without (-) EGCG or EGF as indicated. Total protein was collected at the indicated times and analyzed by western blotting with a $p21^{CIP1}$ monoclonal antibody.

Fig. 8. Effect of EGCG on EGF induced phosphorylation of ERK1/2. A, Growth arrested cells were stimulated with EGF along with the indicated concentration of EGCG. Total protein was collected at 0, 1, 3, and 4 h for analysis by western blotting with antibodies specific to phosphorylated ERK1/2 (top) followed by antibodies that detect both phosphorylated and unphosphorylated ERK1/2 (bottom). B, Cells were treated and total protein analyzed as in A except EGCG was added to 50 uM at 5 hrs where indicated (+).

EFFECT OF EGCG ON PROLIFERATION...

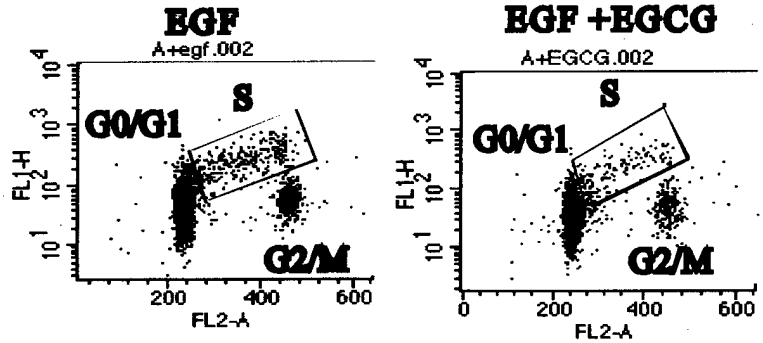
FIGURE 1



AND S PHASE ENTRY

FIGURE 2

A.



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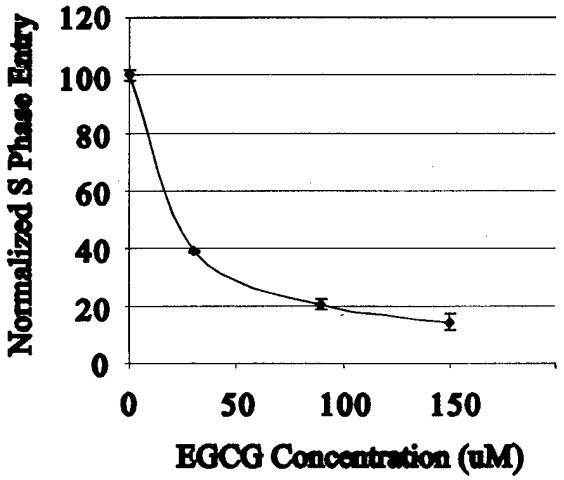
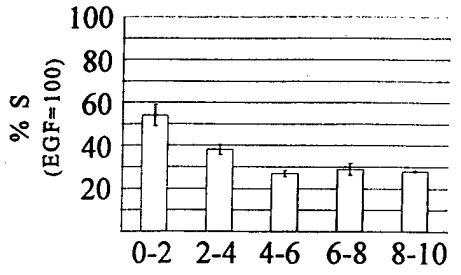


A.



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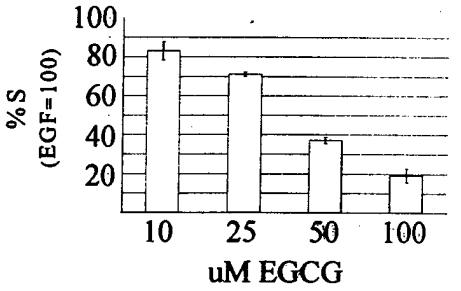

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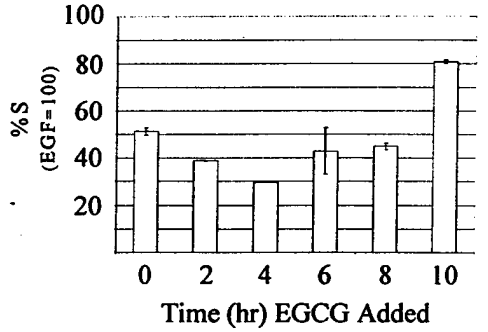

D.

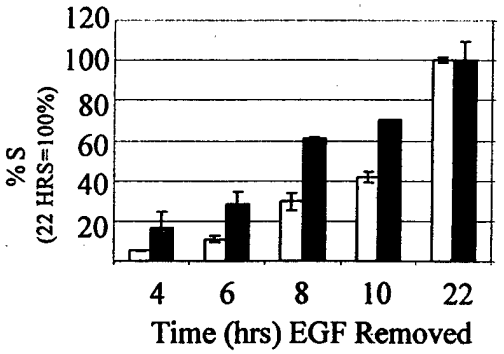

FIGURE 3

EFFECT ON CELL CYCLE REGULATORY PROTEINS

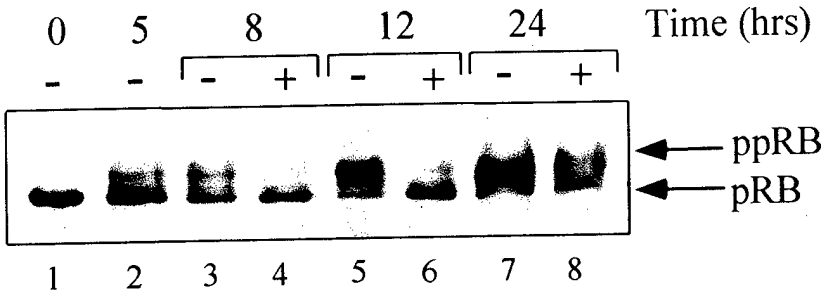

FIGURE 4

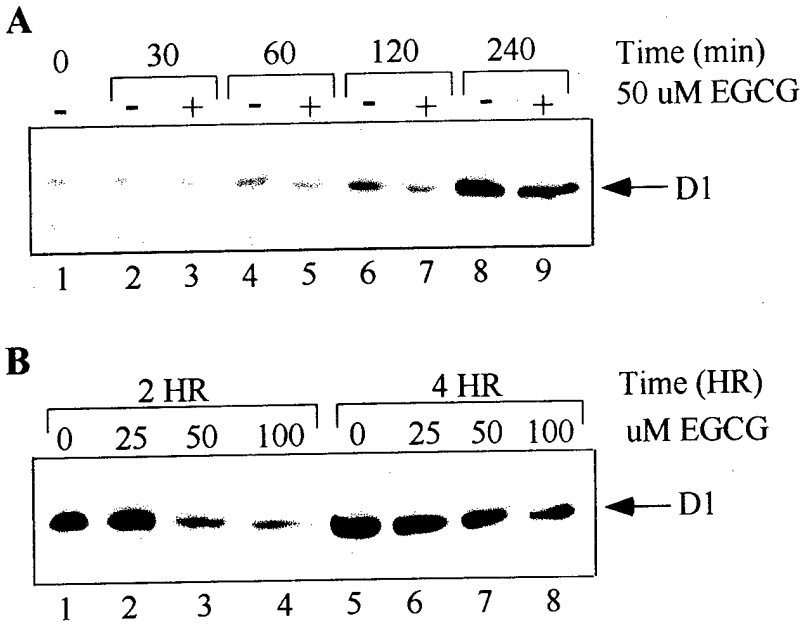

FIGURE 5

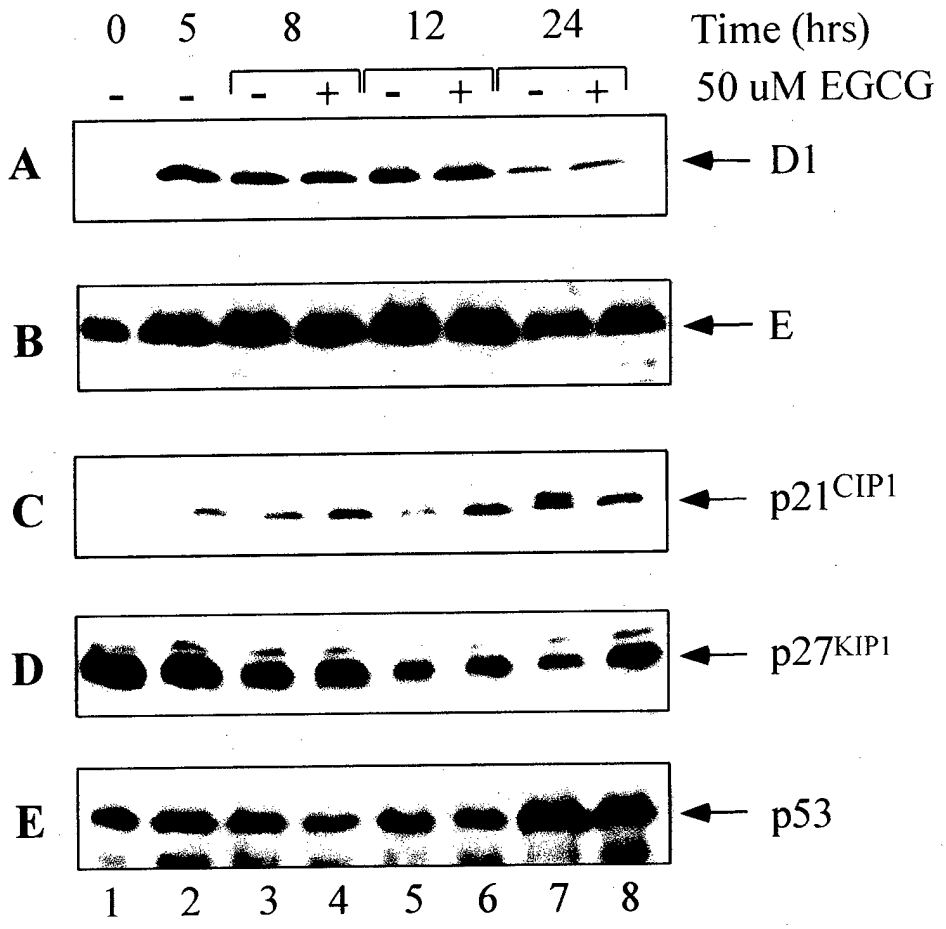
0 5 8 12 24 Time (hrs)
- - - + - + - + 50 uM EGCG
A
D1
B
E
C
p21CIP1
D
p27KIP1
E
p53
1 2 3 4 5 6 7 8

FIGURE 6

EFFECT OF EGCG ON p21 CIP1

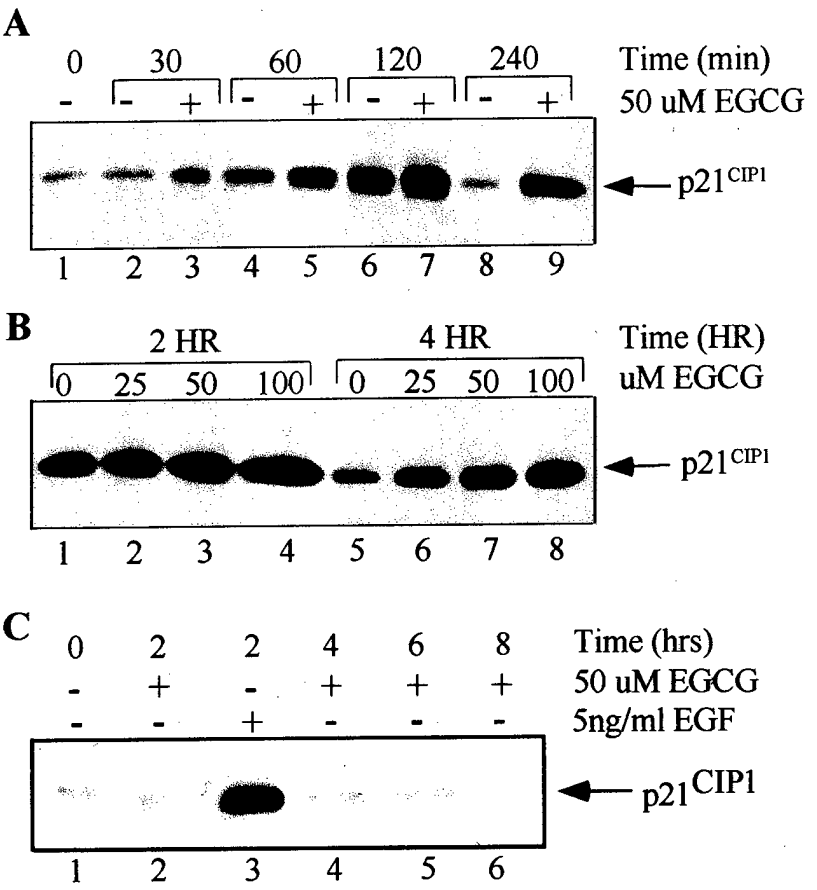

FIGURE 7

ERK1/2 ACTIVATION IN RESPONSE TO EGF AND EGCG DURING G1

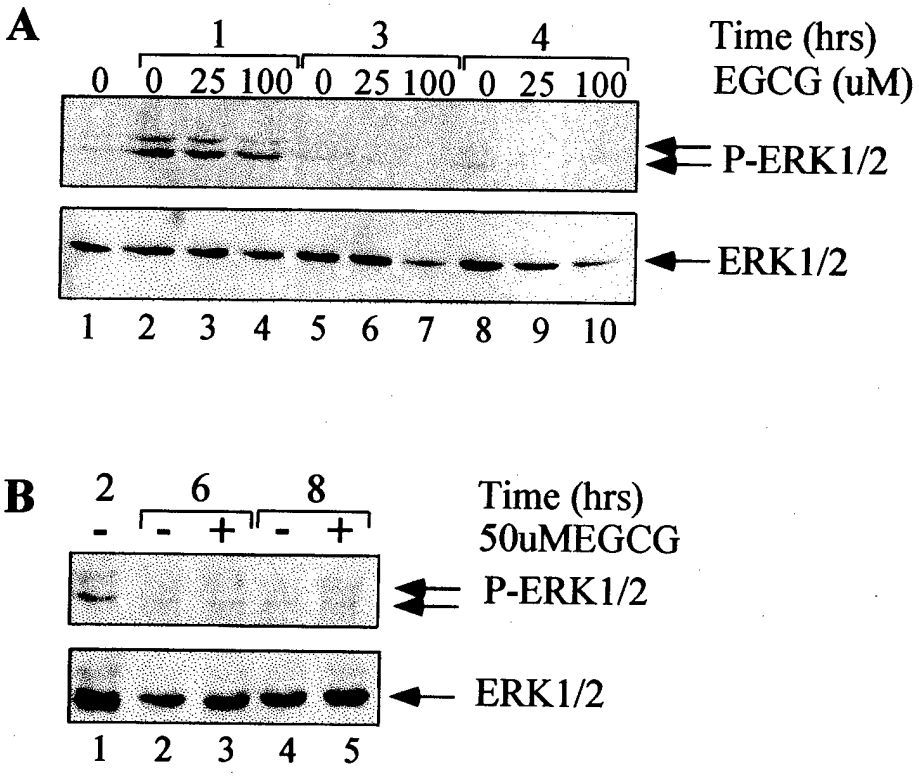

FIGURE 8